IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of MORRE et al — Group Art Unit: 1646

Application N°: 10/522,883 — Examiner: Xie, Xiaozhen

Filed: April 18, 2005

For: IL-7 drug substance, composition, preparation and uses

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Honorable Commissioner of Patents and Trademarks

Washington, D.C. 20231

Sir:

I, Brigitte Assouline, residing 12-14 rue Sébastopol, 92400 Courbevoie, FRANCE, declare and Say:

I am a citizen of France.

I am a graduate of the University of Paris XII-Val de Marne, where I received a Master of Science degree in Cellular and Molecular Biology and of the Ecole Supérieure d'Application des Biotechnologies (ESABIO), where I received a Master degree in Biotechnology. I worked for 8 years in academic research and for the last 10 years, I have worked in biotechnology company in the field of biotechnology, specializing in protein production. .

I am an inventor of the invention claimed in the above-identified patent application.

I read the Office Action from the USPTO, dated November 6, 2008.

I have personally conducted experiments that evidence that the process described by Namen et al (U.S. patent No.5,328,988) does not lead to the production of a pure and fully refolded interleukine 7 (IL-7) conformer.

It is herein demonstrated that Namen does not provide the information necessary to produce a purified IL-7 composition containing more than 98% of the conformer showing the following three disulfide bridges: Cys: 1-4 (Cys2-Cys92); 2-5 (Cys34- Cys129) and 3-6 (Cys47-Cys141).

1. The primary sequence of IL-7 shows a great susceptibility to wrong or uncomplete refolding:

As explained in the patent application and supported by bibliography, the correct and full refolding of a recombinant protein with three disulfide bridges produced either at laboratory scale or for industrial production represents a real challenge for the producers.

Prokaryotic host cells are poorly efficient to refold proteins, often leading the producer to fully denaturate the protein and design a specific step for refolding. In Eukaryotic cells the refolding process is more efficient but can easily become dysfunctional leading to incomplete or wrong refolding even in human cells and this leads to various human pathologies. Naturally at industrial scale and in cells recombined to produce excessive amounts of a specific protein, the cell machinery is unable to fully process the protein and this leads to many protein isoforms, among which the desired conformer should be sorted out by a specific purification process driven by dedicated analytical methods.

Role of the Hinge peptide in IL-7 primary sequence:

In the case of IL-7 this point becomes extremely critical due to the presence of a specific amino acid sequence located upstream of the last alfa helix of the protein : the "Hinge Peptide"

- Pro Ala Ala Leu Gly Glu Ala Gln Pro -

The extreme flexibility of the "HINGE" peptide 101-109 conferred by two Prolines at the extremities and one Glycine 105 in the middle is a specific feature of the IL-7 primary sequence (Fig. 9). It can explain how the positioning of the last helix D, may result in two different refoldings leading to two different conformers. The refolding is probably influenced by drastic conditions encountered during purification process especially during the HPLC purification steps.

The following schematic (Fig. 1) explains the two shapes resulting from two different refolding schemes.

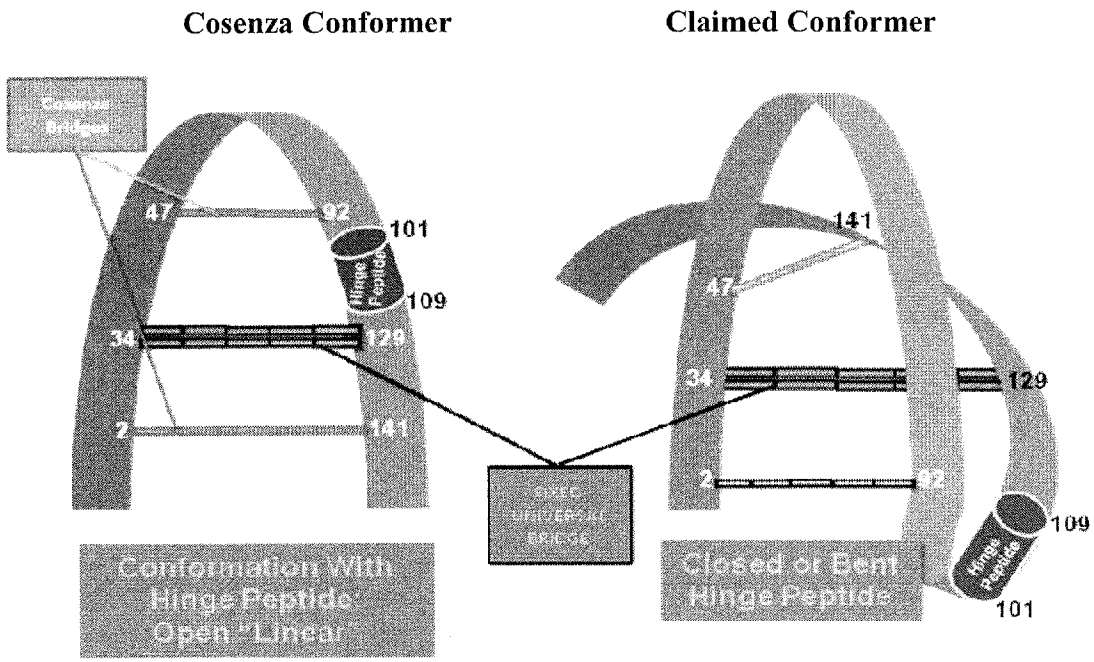


Figure 1: Representation of the structural refolding of IL-7 according to the two different proposed conformers.

Difference originates from the formation of different disulfide bridging scenario, directly linked to the flexibility offered by the unique hinge peptide of the primary structure.

2. The Namen production in prokaryotic cells does not lead to the claimed composition:

In U.S. patent 5,328,988, Namen describes various generic techniques to prepare recombinant IL-7, either from recombined mammalian cells or from microorganisms.

In column 13, from line 9 to line 22, Namen describes his purification process when IL-7 is produced from a **recombined *E. coli* strain**.

2.1. Comparison with the Cosenza process:

This process is easily comparable to the process described by Cosenza et al. (1997)[1] who also use a recombined *E. coli* strain as described on the following pages of their publication:

[1] Cosenza et al. J.Biol.Chem. 1997, 272 (52) pp 32995-33000

p.32996: *Expression and Purification of hIL-7 to Maldi Mass Spectrosopy...*

p.32997: left column *Refolding hIL-7*into a Biologically Active Conformation

The table below shows the similarities of the two processes:

NAMEN	COSENZA	COMPARIBILITY
Recombined *E. coli* strain	Recombined *E. coli* strain	Identical
Cell collection with pellet disruption sonication or freezing	Cell disruption by pellet freezing followed by sonication	Identical
Extraction of cell pellets Preliminary concentration Salting out	Extraction of inclusion bodies and denaturation by 5 M guanidinium chloride	Similar; more details described by Cosenza
Aqueous ion exchange or Size exclusion chromatography	Purification by Size Exclusion HPLC	Identical
Renaturation at 10 to 50µg/ml with 1 to 6M guanidine Chloride	Refolding with glycine L arginine DTT and dialysis	Equivalent; more details described by Cosenza
HPLC final purification	HPLC final purification: HPLC sizing followed by C4 RP HPLC	Similar

After completing his production and purification process, closely similar to Namen description, Cosenza analyzed the resulting purified recombinant human IL-7 for disulfide bonds formation by trypsic digestion of the protein in non denaturing conditions followed by peptide mass determination using MALDI TOF mass spectrometry (see page 32999, bottom left, *Discussion*). In Table II of his paper, Cosenza was able to detect peptides 1 and 23 which are linked by a disulfide bond, thereby establishing the **proof of the Cys3-Cys142 bridge** (equivalent to Cys 2-Cys141 for non methionyl amino terminal IL-7). These two peptides could not be found after digestion in presence of the reducing agent DTT.

This provides proof that one of the processes exemplified by Namen leads to the "Cosenza refolding" which is different from that of the claimed conformer (Cys 2 – Cys 92).

2.2 Role of the purification process:

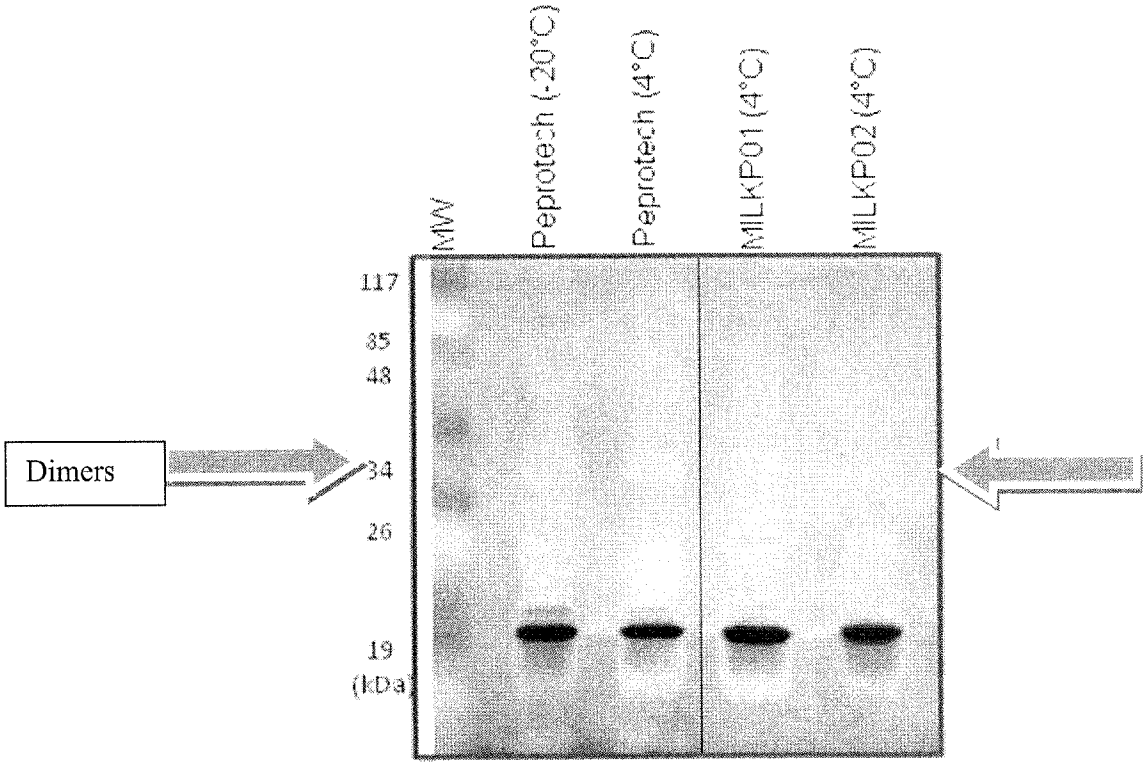
We submitted IL-7 batches produced from recombined E Coli strains of various origins to analysis in non denaturing conditions so as to characterize the presence of misfolded structures and/or multimers or aggregates secondary to intermolecular disulfide bridging.

SDS analysis resolves non covalent protein aggregates, warranting that dimers or multimers detected by this technique are true covalently linked molecules. The link through covalent disulfide bridges is further demonstrated by resolution of these multimers after reduction by any reducing agent susceptible to break diSS bridges..

As shown in Fig 2, the marketed *E coli* derived Preprotech IL-7, purified by HPLC, contains various isoforms among which bands in the 34MW area, reflecting the presence of di SS covalent dimmers resulting from non fully refolded protein.

Another batch produced by Inventors under non fully optimized process, MILKP01 also includes high molecular weights isoforms, while the batch resulting from the optimized process MILKP02 is devoid of these high molecular weight multimers.

Figure 2: *A commercial IL-7 source (Peprotech) and similar IL-7 batches produced in E. coli (MILKP01 or MILKP02 batches) were stored at either -20°C or +4°C and loaded on a SDS PAGE under non reducing conditions.*

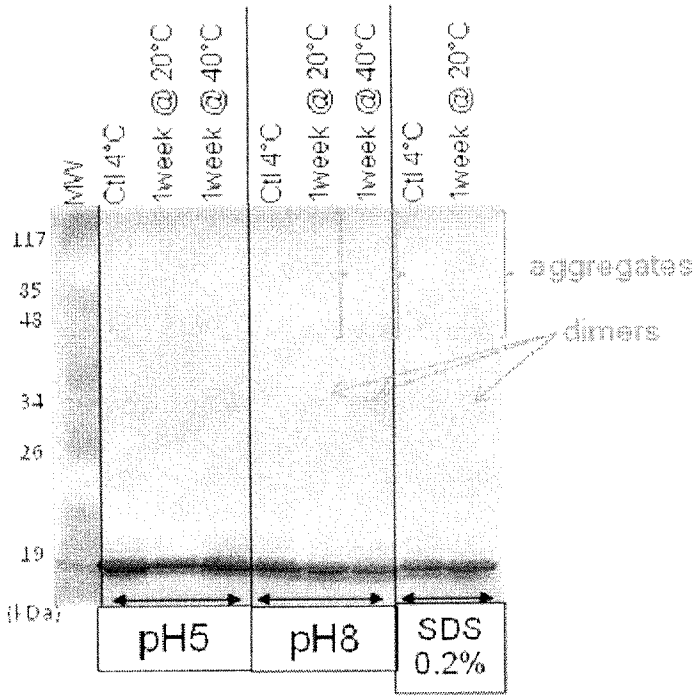


2.3 Role of the formulation to preserve the conformation and stability of rec-hIL-7 batches:

Namen describes an optimal formulation of its purified IL-7 to test the bioactivity: in 100mM NaCl solution buffered at pH8 (column 8 line 50), he also mentions a solution in low SDS for this test at the end of his purification process.

In Fig 3, we show that an IL-7 batch purified from *E. coli* suspension culture is very susceptible to basic pH or low SDS concentration treatments combined to moderate temperatures of incubation during accelerated stabilities studies, further reflecting the lack of full refolding, leaving free cysteins exposed and available for dimer or multimer formation .

Figure 3: *MILKP01 was incubated for 1 week at two different temperatures in the following buffers: Sodium Acetate pH5 (left panel), Phosphate buffer saline pH8 (middle panel) or 0.2% SDS (right panel). Products were separated on SDS PAGE in non reducing conditions. SDS 0.2% and high pHs favor covalent multimerisation and aggregation linked to availability of free cysteins in misfolded proteins.*



3. The Namen production in eukaryotic cells does not lead to the claimed composition:

One could expect from a mammalian host cell a better processing of IL-7 di-SS refolding. However, because of:

- the presence of three disulfide bridges
- the need to produce at industrial scale from a highly producing strain
- the presence of the Hinge Peptide

those eukaryotic cell process cannot warrant the full and correct refolding of all IL-7 molecules produced, unless you drive the purification process with appropriate analytical tools (as in the present invention) to select the claimed conformer.

To demonstrate that Namen was unable to implement this careful selection, we have reproduced a process as close as possible to Namen description.

In US 5,328,988, Namen does not give any method or example to purify a fully refolded conformer of glycosylated recombinant **human** IL-7. Still, he describes a process to purify a glycosylated **murine** IL-7 purified from immortalized bone marrow stromal cells (example 1, columns 16, 17, 18 and 19). Knowing that the murine IL-7 sequence **does not include the Hinge peptide** (Fig 9), this does not teach us how to cope with this very flexible region of IL-7. Consequently Namen is unable to disclose a process warranting full refolding of the human IL-7.

Namen production process is far from teaching us a way to produce a product at industrial scale for large scale and pharmaceutical use: the producing cell is not a characterized cell clone but an immortalized cell line due to tranfection of a pSV3neo plasmid. The downstream processing is cumbersome, with a set of three resin-based chromatographic steps, followed by a set of four HPLC purification steps using two matrixes in double run and finalized through a selection of the bioactive product on a SDS electrophoretic gel from which the product is sliced off and eluted in buffer. The steps of this process, especially the last ones, are barely controllable and fully incompatible with a pharmaceutical use.

Nevertheless, we reproduced the main lines of Namen downstream processing using the supernatant of a recombined CHO clone, one of the most classical expression host cell for recombinant glycosylated proteins.

Chromatographic resins identical or very similar to those used in patent description have been purchased and the process was performed according to patent specifications (Fig. 4). In particular IL-7 containing fractions were collected based on measurement of IL-7 bioactivity of these fractions (not based on a specific purity of a specific IL-7 conformer).

Figure 4: Purification process as defined by Namen *et al.* *Process involves numerous steps, combining usual resin-based chromatographies, repeated HPLCs and ending with a manual purification of product by excision/elution from a SDS PAGE.*

Centrifugation of crude culture harvest. Retrieve supernatant from cell pellet.
Lower the clarified supernatant conductivity through a UltraFiltration Concentration 20x followed by a Dilution 20-fold in water. Final conductivity is = 1mS/cm, a value compatible with optimal passage onto DEAE Sephacel.
DEAE Sephacel pH8, (AEx, capture of contaminants, IL-7 not retained
SP Trisacryl pH5, (CEx, capture/elution of IL-7)
Blue Agarose HP pH8, (Affinity to Cibacron™BlueF3G-A, capture/elution of IL-7)
HPLC purification C4 Vydac
HPLC purification C4 Vydac
HPLC purification C18 Vydac
HPLC purification C18 Vydac
SDS PAGE 12%/ Band Sliced / Elution.

During the capture/elution modes (SP Trisacryl, Blue Agarose) of the resin-based chromatography steps, all elution fractions were analyzed on a SDS Polyacrylamide Gel electrophoresis for the presence of IL-7 and were monitored for potency through the specific Pre B cells IL-7 specific bioassay to cope with Namen's described retain procedure.

During both SP Trisacryl and Blue Agarose steps, bioactive IL-7 is eluted in nearly all fractions and corresponds to a very complex mixture of various proteins (see Fig. 5 & 6A). All the fractions tested in the pre B cell specific bioassay (Namen's claims) exhibit typical and full bioactivity (Fig. 5 & 6B) and as such, according to Namen patent, they should be collected (column 17 line 45, 58, 67) for subsequent HPLC purification.

Figure 5: Purification process, Chromatographic step 2 (SP Trisacryl), SDS PAGE analysis of eluted fraction and screen for bioactivity.

Elution fractions were pooled according to SDSPAGE results and assayed for potency in an IL-7 specific bioassay. All fractions were found to exhibit bioactivity: a sigmoid curve with EC50 comparable to a reference standard.

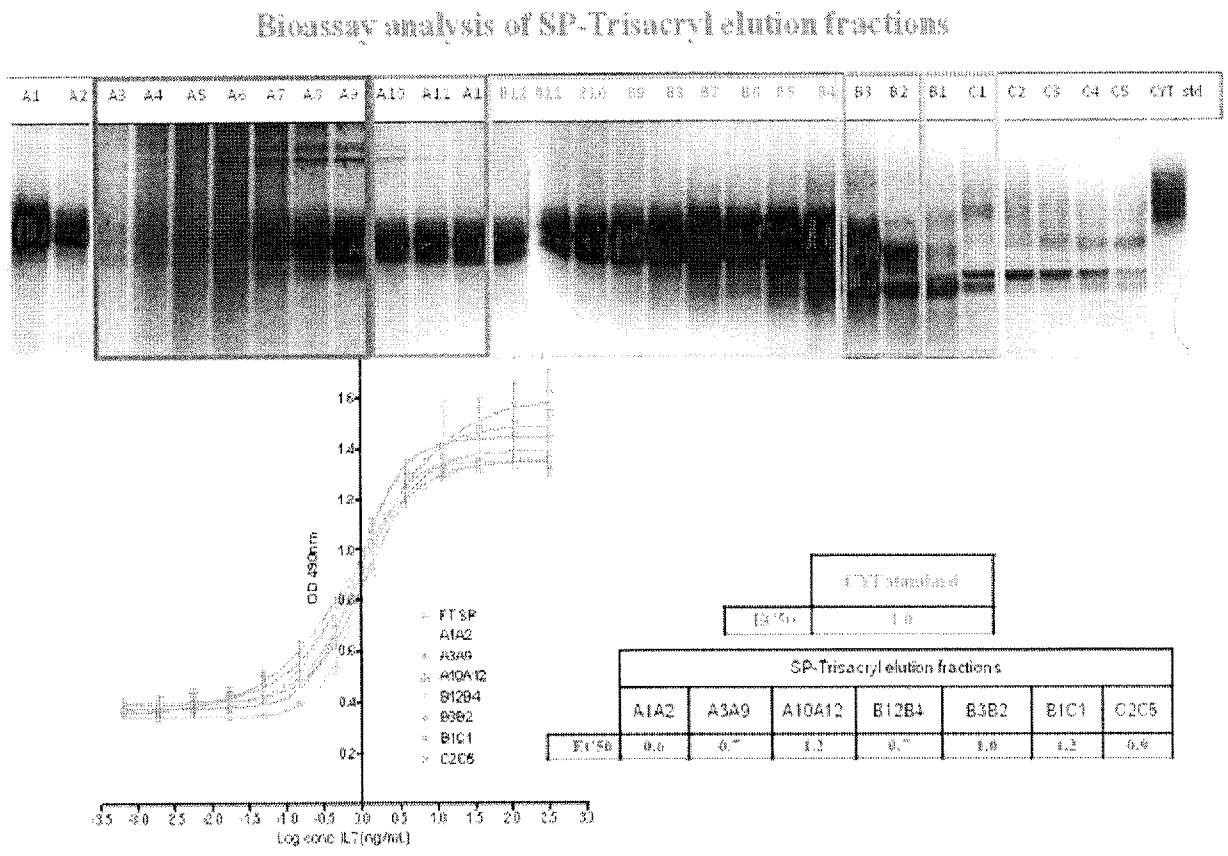
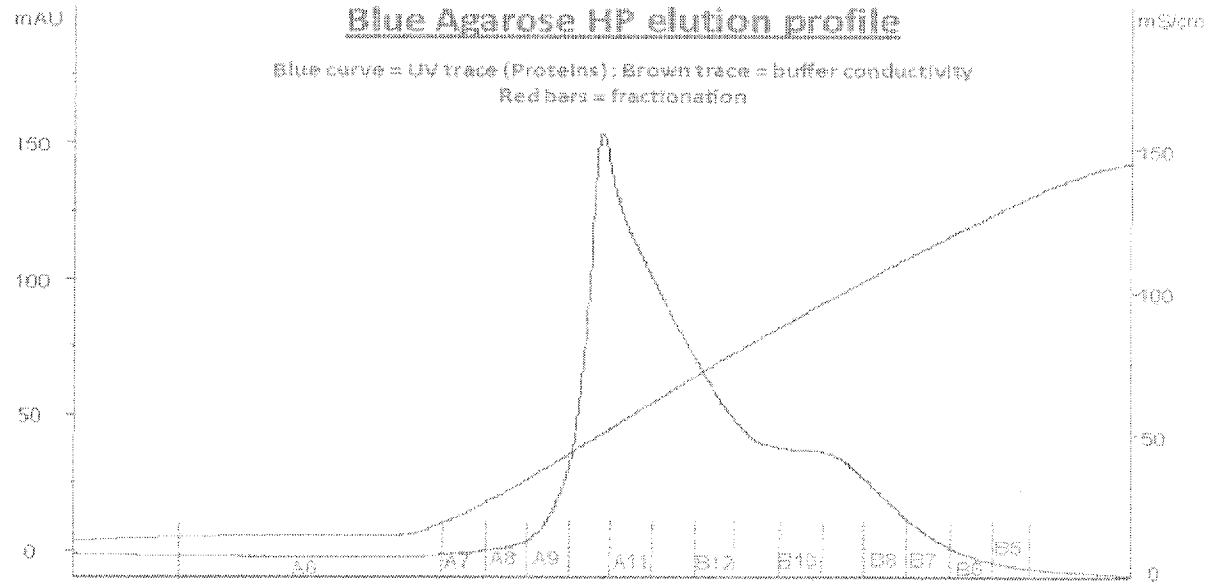


Figure 6A: Chromatographic step 3 (Blue Agarose HP) of purification process. *Column elution profile and SDS PAGE Analysis of eluted fractions.*



SDS analysis of BLUE AGAROSE fractions

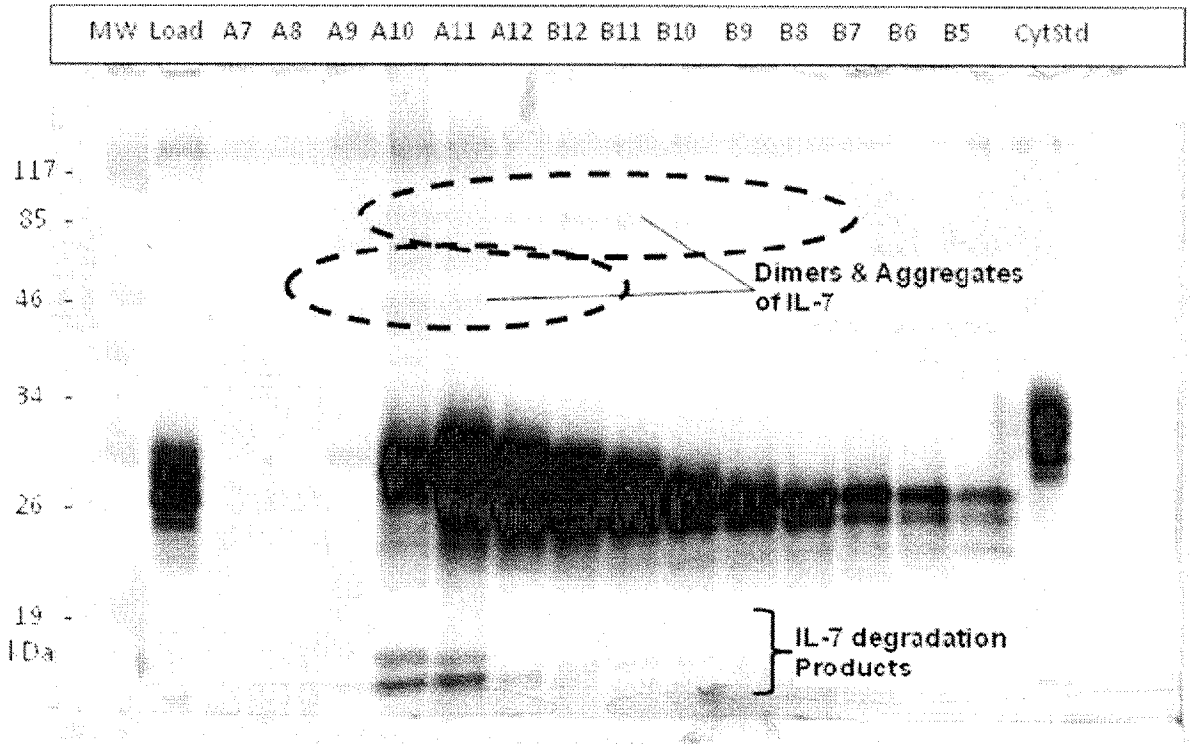
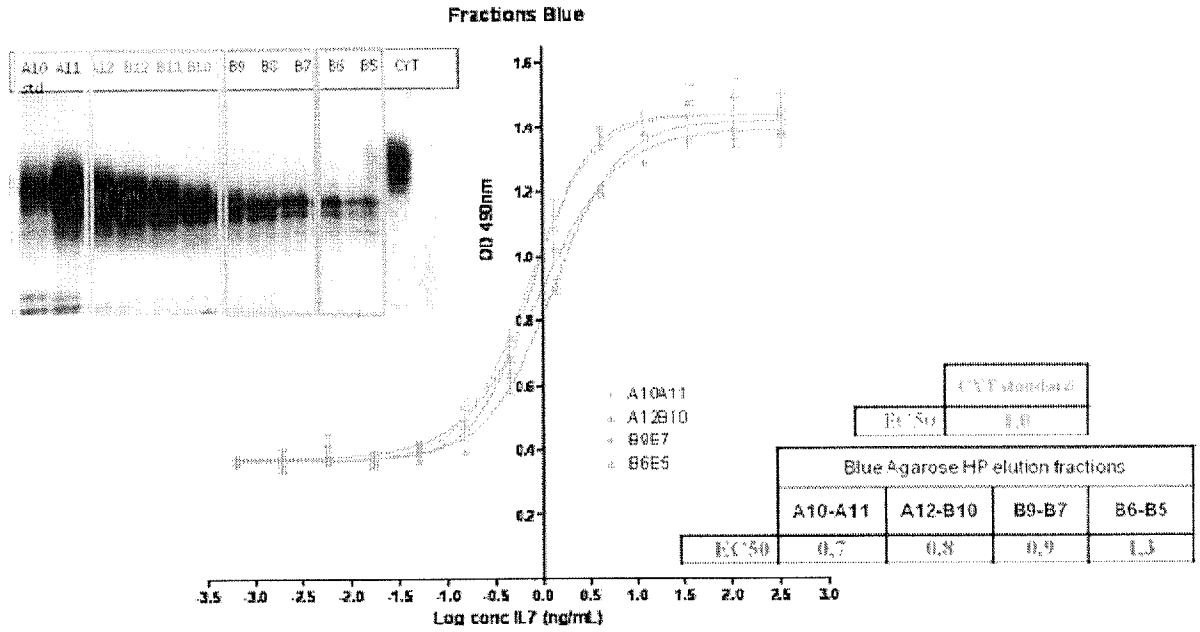


Figure 6B: Chromatographic step 3 (Blue Agarose HP) of purification process, screen of elution fractions for bioactivity.

Elution fractions were pooled according to SDSPAGE results and assayed for potency in an IL-7 specific bioassay. ***All fractions were found to exhibit bioactivity*** *with a sigmoid curve showing EC50 comparable to a reference standard.*

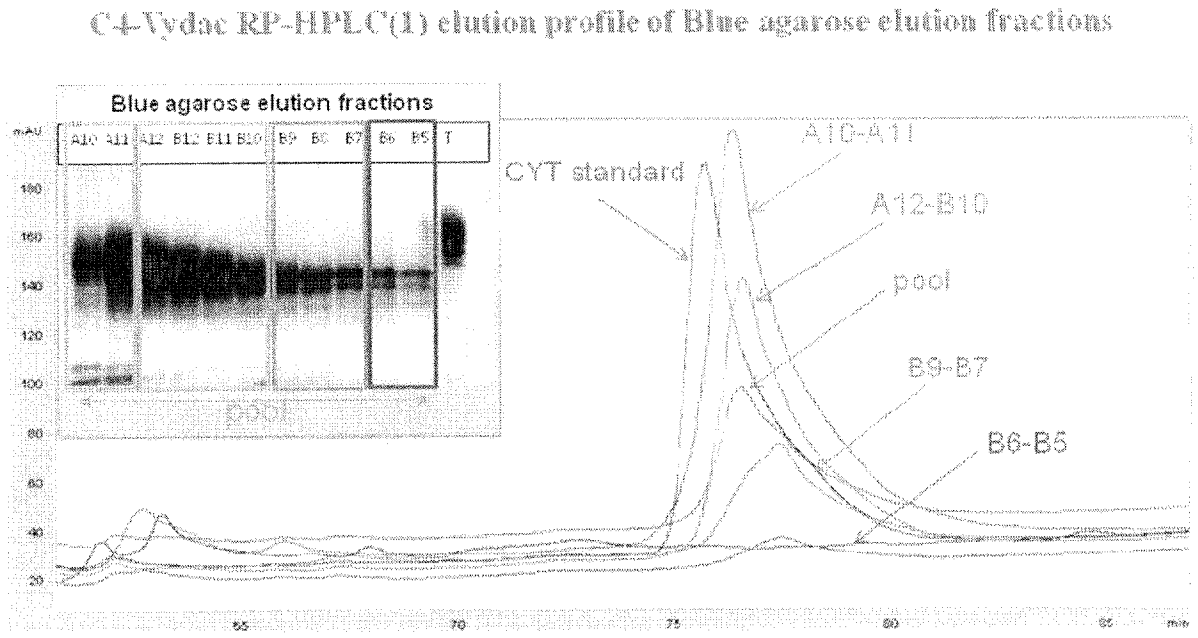


Then the bioactive fractions collected from these two steps were purified on C4 Vydac HPLC column.

Again all fractions selected for bioactivity gave each a peak in HPLC so that the bioactivity criteria is not adequate to select a specific IL-7 conformer. The Pooll of all fractions also gave a single peak.

Figure 7: Purification process, HPLC purification after all Chromatographic steps.

Blue Agarose HP elution fractions exhibiting IL-7 activity were pooled and loaded onto a C4 RP-HPLC column according to the protocol described by Namen et al. General pool as well as selected fraction all exhibit a single peak by HPLC, with no sorting out of IL-7 versus impurities. **All the peaks will be collected for subsequent purification.**



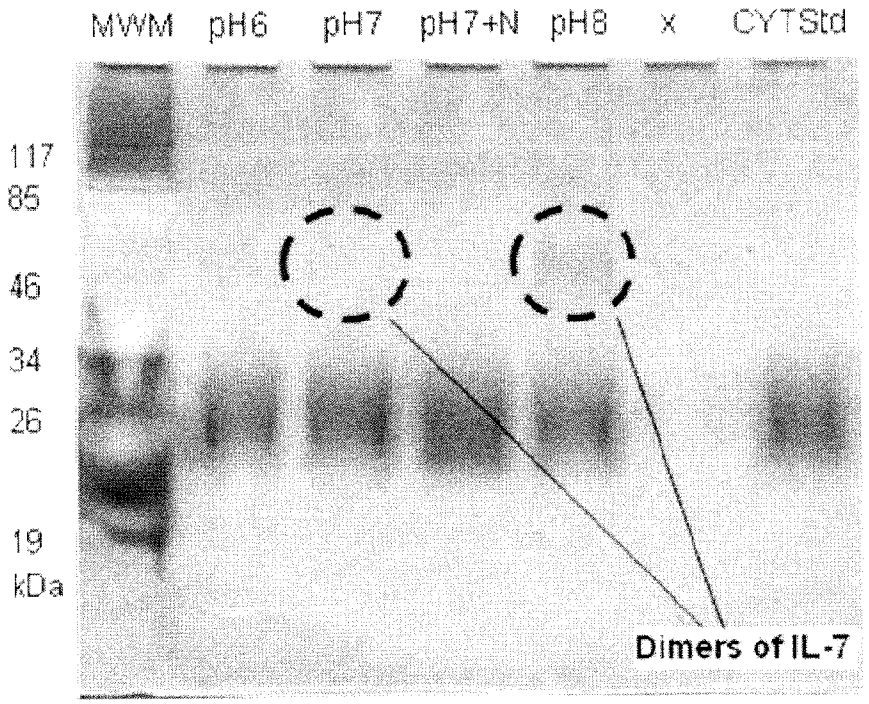
The collection of these complex mixtures could never lead to a highly purified conformer after completion of the subsequent double HPLC chromatographic steps (Fig. 7).

Importantly Namen stipulates that at the end of his purification process, he detects various bands by SDS gel analysis (column 19, line 13) "a number of proteins were still present", among which a minor "faint" band above the major band was associated with bioactivity.

This clearly demonstrates that the Namen protein, although bioactive, is not a highly purified specific IL-7 conformer compatible with lack of immunogenicity.

Furthermore, when testing the bioactivity of his protein, Namen uses a 100mM NaCl solution at pH8 (column 18 line 50). We have tested various buffers/pH conditions to find an optimal stability of the recombinant protein, avoiding the production of dimers through reduction of free cysteins (Fig.8). In the SDS gel below, we show that buffers having an acidic pH like 5 or 6 minimize or block the formation of such covalent dimers, while pH8 is the optimal pH to promote dimers' formation. In an SDS gel electrophoresis, covalent dimers may only result from inter-molecular bridging of IL-7 molecules through free cysteins, cysteins not engaged in intra-molecular bridge of the claimed conformer, since treatment of the SDS gel by reducing agents immediately resolves those covalent multimers (reflected by disappearance of the high MW bands)

Figure 8: Susceptibility of IL-7 to pH.
Samples of the recombinant glycosylated hIL-7 clinical batch were incubated 1 week at +40°C in Phosphate buffers at pH conditions specified on top of each lane. +N = buffer was supplemented with 50 mM Arginin ; CYT Std = IL-7 reference standard stored at -20°C at pH=5.



CONCLUSION

Namen does not disclose a process that could lead to a purified claimed conformer necessary to prepare a non-immunogenic IL-7 composition.

As shown in section 2 above, the production of IL-7 according to Namen, in prokaryotic cells, does not lead to the right conformer purity.

As shown in section 3 above, the production of IL-7 according to Namen, in eukaryotic cells, does not lead to the right conformer purity either.

By selecting his fractions on the base of specific bioassay testing, Namen drives the process to capture a maximum of bioactivity but he never proposes or implements any method able to select the claimed conformer.

Inventors have shown that a pharmaceutical composition of IL-7 for therapeutic use should contain at least 98% of said conformer to avoid the generation of anti-IL-7 immunogenicity in patients.

I declare further that all statements made herein are of our own knowledge true and all statements made on information or belief are believed to be true: further that all statement were made with the knowledge that willful false statements and like so made are punishable by fine or imprisonment, or both, under § 1011 of title 18 of the United States code and that suchwillful false statements may jeopardize the validity of the application and any patent issuing thereon. Further Declarants sayeth not.

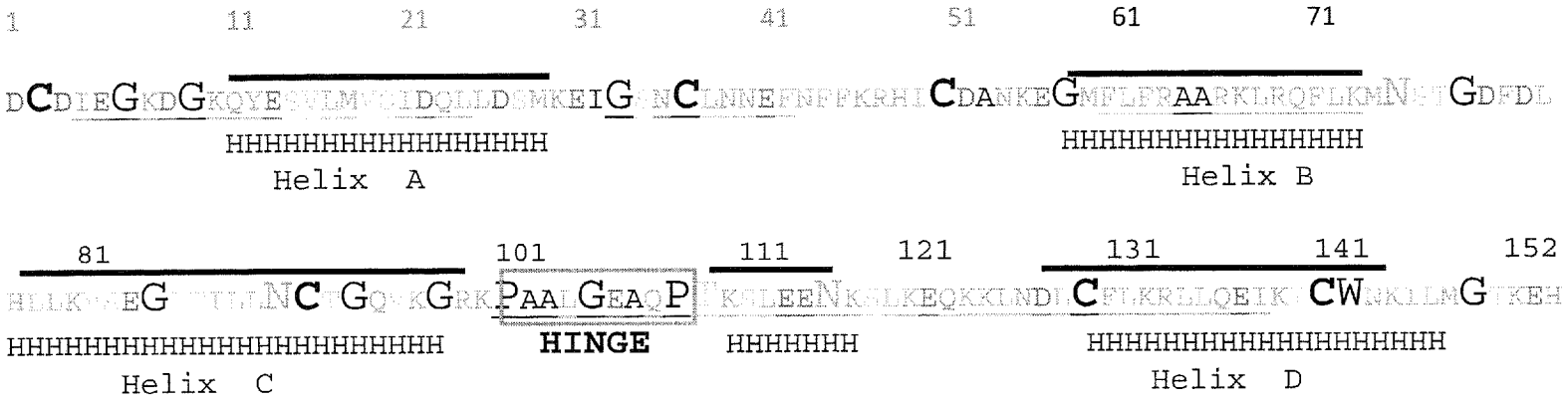
April 3, 2009

Date

Brigitte Assouline

Figure 9: Primary sequence of human Interleukin -7 and sequence alignment between the human and murine primary sequence. In both pictures, the box represent the position of the hinge peptide showing the absence of this Hinge Peptide sequence in the murine IL-7

Human INTERLEUKIN-7 SEQUENCE



C : cysteins

Human /Murine INTERLEUKIN-7 SEQUENCES alignment

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           1         10         20         30         40         50         60         70         80
Human IL-7  DCDIEGKDGK QYESVLMVSI DQLLDSMKEI GSNCLNNEFN FFKRHICDAN KEGMFLFRAA RKLRQFLKMN STGDFDLHLL
Murine IL-7 ECHINDKEGK AYESVLMISI DSL-DKMIGT DSNCENNETN FFRKHVCDDT KEAAFLNRAA RKLKQFLKMN ISEEFNVHLL

           81        90         100        110        120        130        140            152
Human IL-7  KVSEGTTILL NCTGQVKGRK PAALGEAQPT KSLEENKSLK EQKKLNDLCF LKRLLQEIKT CWNKILMGTK EH
Murine IL-7 TVSQGTQILV NCTSK----- ---------- ----EEKNVK EQKK-NDACF LKRLLREIKT CWNKILKGST
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